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ASSOCIATIONS BETWEEN PERFLUOROALKYL COMPOUNDS AND IMMUNE AND CLINICAL CHEMISTRY PARAMETERS IN HIGHLY EXPOSED BOTTLENOSE DOLPHINS (*TURSIOPS TRUNCATUS*)

PATRICIA A. FAIR,*† TRACY ROMANO,‡ ADAM M. SCHAEFER,§ JOHN S. REIF,‖ GREGORY D. BOSSART,# MAGALI HOUDE,‖†† DEREK MUIR,‡‡ JEFF ADAMS,§§ CHARLES RICE,‖‖ THOMAS C. HULSEY,## AND MARGIE PEDEN-ADAMS†††

†National Oceanic and Atmospheric Administration, National Ocean Service, Center for Coastal Environmental Health and Biomolecular Research, Charleston, South Carolina, USA
‡Mystic Aquarium and Institute for Exploration, Mystic, Connecticut, USA
§Harbor Branch Oceanographic Institute at Florida Atlantic University, Ft. Pierce, Florida, USA
‖Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, Colorado, USA
#Georgia Aquarium, Atlanta, Georgia, USA
‖††Environment Canada, Centre Saint-Laurent, Montréal, Canada
‡‡Aquatic Ecosystem Protection Research Division, Environment Canada, Burlington, Ontario
§§National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Silver Springs, Maryland, USA
‖‖Department of Biological Sciences, Clemson University, Clemson, South Carolina, USA
##Medical University of South Carolina, Charleston, South Carolina, USA
†††Harry Reid Center for Environmental Studies, University of Nevada Las Vegas, Las Vegas, Nevada, USA

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Abstract—Perfluoroalkyl compounds (PFCs) are ubiquitous, persistent chemical contaminants found in the environment, wildlife, and humans. Despite the widespread occurrence of PFCs, little is known about the impact these contaminants have on the health of wildlife populations. The authors investigated the relationship between PFCs (including ∑perfluorocarboxylates, ∑perfluoroalkyl sulfonates, perfluorooctane sulfonate, perfluorooctanoic acid, and perfluorodecanoic acid) and the clinocopathologic and immune parameters in a highly exposed population ($n = 79$) of Atlantic bottlenose dolphins (mean ∑PFCs = 1970 ng/ml; range 574–8670 ng/ml) sampled from 2003 to 2005 near Charleston, South Carolina, USA. Age-adjusted linear regression models showed statistically significant positive associations between exposure to one or more of the PFC totals and/or individual analytes and the following immunological parameters: absolute numbers of CD2+ T cells, CD4+ helper T cells, CD19+ immature B cells, CD21+ mature B cells, CD2/CD21 ratio, MHCII+ cells, B cell proliferation, serum IgG1, granulocytic, and monocytic phagocytosis. Several PFC analyte groups were also positively associated with serum alanine aminotransferase, gamma-glutamyltransferase, creatinine, phosphorus, amylase, and anion gap and negatively associated with cholesterol levels, creatinine phosphokinase, eosinophils, and monocytes. Based on these relationships, the authors suggest that the PFC concentrations found in Charleston dolphins may have effects on immune, hematopoietic, kidney, and liver function. The results contribute to the emerging data on PFC health effects in this first study to describe associations between PFCs and health parameters in dolphins. Environ. Toxicol. Chem. 2013;32:736–746. © 2013 SETAC

Keywords—Perfluoroalkyl compounds Immunology Hematology Serum chemistry Bottlenose dolphins

INTRODUCTION

Perfluoroalkyl compounds (PFCs) constitute a general class of highly stable chemicals that include several commonly used perfluorinated chemicals [1]. They resist biodegradation, photooxidation, and hydrolysis and have properties of water and oil repellency and stain resistance. As such, they have been used widely in industrial and consumer products as surface coatings and protectants due to their unique surfactant properties [2,3]. The two most widely used and investigated PFCs are perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) [1,2]. As a consequence of their widespread use and persistence, biomonitoring studies have shown that these chemicals are present in humans and wildlife worldwide [3]. Perfluoroalkyl compounds have been detected globally in a wide variety of marine and terrestrial animals and humans [4,5]. Fish-eating species such as mink, bald eagles, and marine mammals often exhibit high PFC concentrations [4,6]. In fact, some of the highest PFC levels reported in marine mammals have been reported in bottlenose dolphins (*Tursiops truncatus)* living in the estuarine waters of Charleston, South Carolina, USA, an urban area of the southeast United States [7,8]. Perfluoroalkyl compounds are persistent and bioaccumulate, binding to plasma proteins and fatty acids in the circulation and are stored in the liver [9].

Widespread environmental and human health concerns regarding PFC compounds, especially PFOS and PFOA, have resulted in a large body of toxicological, epidemiological, and environmental information [2,9]. Toxicity studies of PFOS in laboratory animals have reported alterations in liver physiology and hepatomegaly in the rat, mouse, and cynomolgus monkey with induction of lipidemia and peroxisomal fatty acid β-oxidation, alterations in serum cholesterol, increases in serum alanine aminotransferase (ALT), and wasting syndrome [9–11]. Gestational exposure to PFOS causes developmental, endocrine, and reproductive effects including increased incidence of prenatal mortality, low birth weights, structural defects, and developmental delays [12]. In addition, several studies indicated that the immune system may be a target for PFCs in both

* To whom correspondence may be addressed (pat.fair@noaa.gov).
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adult and developmental rodent models [13,14]. Evidence that PFCs might alter immune function resulting in increased susceptibility to infectious disease was observed in recent studies in which PFOS increased susceptibility to influenza A virus in rodents [15]. Furthermore, serum PFC concentrations in children were associated with reduced production of antigen-specific antibodies following immunization [16].

Increasing concern for public health resulted in the Centers for Disease Control and Prevention including PFCs in national biomonitoring programs to track the presence of these chemicals in humans and record associated adverse events [17]. Although human health effects from PFCs at low environmental exposures are largely unknown, several studies indicated that serum PFOA and PFOS concentrations in the general population are associated with increased risk of infertility, reduced humoral immune response, and low birth weights [18,19]. Furthermore, occupational exposures to PFCs have been associated with a wide range of adverse health effects including elevated serum hepatic enzymes, uric acid, and cholesterol and decreased serum IgE and IgA [20–23].

Despite the ubiquitous occurrence of PFCs and their known effects in laboratory animals, little is known regarding the potential health impacts of these contaminants on wildlife. One study in marine mammals showed that concentrations of both PFOA and PFOS were significantly higher in sea otters (*Enhydra lutris*) that died from infectious disease compared to those that died from noninfectious causes [24]. Our previous studies have found high PFC concentrations in plasma from bottlenose dolphins inhabiting the coastal areas of Charleston, South Carolina, [8] similar to those of occupationally exposed humans (mean PFOS concentration of 2.2 ppm) [25]. The highly exposed dolphins from the Charleston estuarine areas provide an opportunity to examine the potential health effects that might be related to their high PFC levels. Thus, the purpose of the present study was to investigate the relationships between concentrations of PFC pollutants in dolphins inhabiting the Charleston area with immune and clinicopathological parameters.

MATERIALS AND METHODS

Study population

Dolphin capture–release health assessments were conducted from 2003 to 2005 in the estuarine coastal areas of Charleston, South Carolina under National Marine Fisheries Service permit No. 998 to1678 issued to Gregory Bossart, and Institutional Animal Care and Use Committee approval through Harbor Branch Oceanographic Institution. Detailed information pertaining to the study site, methods for capture, sampling, and release are described elsewhere [26]. For the present study, we evaluated associations between PFC concentrations and immunological, hematological, and serum chemistry parameters in 79 dolphins from Charleston.

Blood samples were drawn from the periarterial rete in the flukes through a butterfly catheter. Whole blood was collected in vacutainer tubes containing sodium heparin or ethylenediamine-tetraacetic acid (EDTA) as anticoagulants and serum was collected in 10 ml separator vacutainer tubes (Becton Dickinson). Samples for clinical chemistry were held for 30 to 40 min and centrifuged at 1,233 *g* for 15 min. Fibrin clots were removed and serum transferred to plastic tubes were sent to Cornell University Veterinary Diagnostic Laboratory in Ithaca, New York, USA, along with EDTA tubes for standard hematological tests. Samples for immunological tests were kept cool and shipped overnight to the Mystic Aquarium and Institute for Exploration in Mystic, Connecticut, USA, the Medical University of South Carolina in Charleston, South Carolina, and Clemson University in Clemson, South Carolina. Plasma was collected from heparinized blood, transferred into polypropylene cryovials, and frozen at −80°C for PFC analysis and to measure lysozyme activity. Age was determined by counting postnatal dentine layers in an extracted tooth [27].

Exposure assessment

Concentrations of PFCs in plasma were determined at the Environment Canada's Laboratories in Burlington Ontario. Sample extraction, analysis, and quality control procedures are detailed by Houde et al. [7]. Plasma samples were stored at –80°C and PFCs were quantified using high-performance liquid chromatography with negative electrospray tandem mass spectrometry (HPLC-MS/MS). The PFC analytes and precursors analyzed were as follows: PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUA), perfluorododecanoic acid (PFDoA), perfluoro-n-tetradecanoic acid (PFTriA), perfluorotetradecanoic acid (PFTA), 8:2 and 10:2 fluorotelomer acid (FTCA), 8:2 and 10:2 fluorotelomer unsaturated acid (FTUCA), perfluorohexane sulfonic acid (PFHxS), PFOS, perfluorooctanesulfonamide (PFOSA), perfluoroheptanoic acid (PFHpa), and perfluorodecane sulfonate (PFDS). Data quality assurance and control measures included both field and laboratory blanks, matrix spikes, and standard reference material injection every 10 samples to monitor changes in the sensitivity of the instrument.

Hematology, serum chemistry, and protein electrophoresis

The methods for hematology, protein electrophoresis, and serum chemistry analyzed by the Animal Health Diagnostic Center, Veterinary College, Cornell University have been described previously [26]. Briefly, differential leukocyte counts were performed by microscopic examination of modified Wright-Giemsa stained blood smears (Bayer Healthcare). A microhematocrit tube was centrifuged for 5 min at 13,700 *g*, and the manual hematocrit was interpreted by visual inspection against a standard calibration. Serum protein electrophoresis was performed on an automated analyzer (Rapid Electrophoresis, Helena Laboratories). Hemoglobin, red blood cell count, red cell distribution width, mean corpuscular platelet volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, total platelets, and mean platelet volumes were determined by an automated analyzer (Bayer ADVIA 120, Bayer Diagnostics). The concentrations of serum chemistry analytes were determined with an automated analyzer (Hitachi 917, Roche). Fibrinogen concentration was determined in EDTA anticoagulated blood by the method of Schalm using heat precipitation.

Immune assays

The methods, sources of chemicals, reagents, and antibodies for immune assays in dolphin blood samples were described previously [28]. The concentration of IgG1 was determined in serum using a sandwich capture enzyme-linked immunosorbent assay with two monoclonal antibodies as previously described [29]. Peripheral blood leukocytes to assess proliferation, natural killer cell activity, superoxide production, and immunophenotyping were isolated from whole blood by a slow spin technique as previously described [28] with minor modifications, counted and assessed for viability, and subsequently diluted as described for each endpoint.

Immunophenotyping. Lymphocyte subsets were labeled and analyzed as previously described [30]. Briefly, 1×10^6 cells/ml were labeled with 50 μl of monoclonal supernatant for 30 min at 4°C followed by fluorescein isothiocyanate conjugated affinity purified goat anti-mouse F(ab)′2 IgG1 for 30 min at 4°C in the dark. Cells were analyzed using flow cytometry on an LSR flow cytometer (BD Biosciences).

Phagocytosis. The percentage of phagocytosis for granulocytes and monocytes was determined using a modification of the technique previously described [31]. Briefly, cells were incubated with 10 μl of 2.1×10^9 units/ml heat-killed *Staphylococcus aureus* labeled with 100 μg/ml of propidium iodide added at 25:1 bacteria:cell ratio and then incubated in a shaking 37°C water bath for 0 and 75 min. Phagocytosis was stopped by adding 10 μl of 1 mM N-ethylmaleimide and red blood cells were lysed by the addition of 1 ml of lysis buffer. Cells were resuspended in paraformaldehyde and analyzed on an LSR flow cytometer.

Lysozyme activity. Serum lysozyme activity was assessed using slight modifications of a standard turbidity assay [32]. A solution of *Micrococcus lysodeikticus* was prepared fresh daily (50 mg of the lyophilized cells in 100 ml, 0.1 M phosphate buffer, pH 5.9) and added to the sample and standard wells. Hen egg lysozyme (Sigma) used to produce a standard curve. Plates were read at absorbance of 450 nm on a spectrophotometer (SpectraCount, Packard) immediately and again after 5 min. Absorbance unit values were converted to hen egg lysozyme concentration (μg/μl) via linear regression of the standard curve.

Mitogen-induced lymphocyte proliferation. The lymphocyte proliferation response was measured using methods optimized previously [28]. Isolated viable peripheral blood leukocytes (1×10^5 cells/well) were incubated in 96-well plates with 2.5 μg/ml concanavalin A (Con A; type IV-S; a T-cell mitogen), 120 μg/ml lipopolysaccharide (LPS; *E. coli* 055:B5; a B-cell mitogen), or cell media without mitogen (unstimulated wells) in triplicate. Following a 96-h incubation period at 37°C and 5% CO_2, 0.5 μCi of tritiated thymidine was added to each well. Cells were harvested and analyzed using a Packard Top Count-NXT scintillation counter.

Natural killer cell activity. Natural killer cell activity was assessed via an in vitro cytotoxicity assay using ^{51}Cr-labeled Yac-1 cells as described previously with minor modifications [28]. Peripheral blood leukocytes (1×10^7 nucleated cells/ml) and ^{51}Cr-labeled Yac-1 cells were prepared, in triplicate, in ratios of 100:1, 50:1, and 25:1. After a 6-h incubation at 37°C and 5% CO_2, plates were centrifuged and 25 μl of supernatant transferred to a 96-well plate containing solid scintillant (LumaPlate). Plates were air dried overnight, and then counted on a Packard Top Count-NXT.

Data Analyses

Previous analyses showed significantly higher PFC concentrations in dolphins from the Charleston, South Carolina coastal areas compared to other areas along the coastal United States [7,8]. Therefore, we examined associations between PFCs and health parameters in only the highly exposed Charleston dolphins to minimize confounding from differences in other exposures at the respective sites. The concentrations of ∑PFCs, ∑perfluorocarboxylates (PFCA) and ∑perfluoroalkyl sulfonates (PFSA) in dolphin plasma were calculated, assessed for normal distribution, and log transformed as needed to meet test assumptions. The ∑PFCs included PFDA, PFDS, PFDoA, PFHpA, PFHxS, PFNA, PFOA, PFOS, PFOSA, PFTA, PFTriA, and PFUA. Perfluorocarboxylates included PFOA, PFNA, PFDA, PFUA, PFDoA, PFTriA, PFTA, and PFPA. Perfluoroalkyl sulfonates included PFOS, PFOSA, PFDS, and PFHxS. Regression analyses were conducted for PFOS and PFOA, the two PFCs of most interest, and for PFDA, which had the second-highest concentration following PFOS (Table 1). Descriptive statistics included arithmetic means with ranges and standard deviations for each analyte. The percentage of nondetectable samples were 0% for all analytes with the following exceptions; PFDoA, PFTriA, PFDS, and PFTA which were 2.5, 21, 28, and 48%, respectively. Nondetectable concentrations were set to the minimum detection limit divided by $\sqrt{2}$ [33]. The minimum detection limit for all PFC analytes was 0.5 ng/g, except for PFDA and PFUA, which were 0.8 ng/g. Sexual maturity in bottlenose dolphins has been categorized from 5 to 12 years for females and 10 to 13 years for males [34]. Therefore, adults were defined as females age 7 and older and males age 10 and older. Juveniles were categorized as less than these ages.

Perfluoroalkyl compound concentrations were previously reported to decrease significantly with age in these dolphins [8]. Thus, age-adjusted linear regression models were used to determine the relationships between log-PFCs levels and the immune, hematology, and serum chemistry parameters of interest. Log-PFC concentrations were not significantly different between genders using an independent samples *t* test. Therefore, gender was not included as a covariate. Stratified gender- and age-specific analyses for PFOS were examined for two reasons. Perfluorooctane sulfonate was the predominant PFC found in the present study, accounting for approximately 70% of the total burden of PFCs. Second, gender-specific immune effects of

Table 1. Descriptive statistics (means, standard deviations [SD], and ranges) for plasma concentrations (ng/ml) of ∑perfluorochemicals (PFC), ∑perfluoroalkylsulfonates (PFSA), and ∑perfluoroalkylcarboxylates (PFCA), perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and perfluorodecanoic acid (PFDA) in dolphins by sex and age from Charleston, South Carolina, USA.

Variable	∑PFC Mean (SD) Range	∑PFCA Mean (SD) Range	∑PFSA Mean (SD) Range	PFOS Mean (SD) Range	PFOA Mean (SD) Range	PFDA Mean (SD) Range
Total ($n = 79$)	1970 (1380)	204 (184)	1530 (1080)	1420 (969)	55.1 (75.4)	177 (131)
	574–8670	26–845	356–6792	317–6257	0.5–561	41–667
Sex						
Male ($n = 47$)	2180 (1690)	221 (194.23)	1700 (1370)	1560 (1220)	54.7 (58.6)	201 (145)
	586–8670	31–783	356–4725	317–4113	3–561	48–667
Female ($n = 32$)	1830 (1120)	193 (178)	1420 (884)	1320 (754)	54.7 (85.5)	160 (119)
	574–5619	26–845	512–7059	491–6257	0.5–296	41–620
Age class						
Juvenile ($n = 35$)	2395 (1720)	252 (192)	1940 (1380)	1770 (1240)	79 (97.3)	226 (153)
	595–8670	31–845	356–7059	317–6257	3–561	48–667
Adult ($n = 44$)	1491 (752)	153 (150)	1170 (556)	1100 (519)	33.4 (41)	128 (76)
	574–3822	26–783	418–3231	395–2912	0.5–275	41–368

PFOS exposure have been described in laboratory animals including an increased natural killer cell activity in males [35,36]. We examined the effects of removing dolphins classified as diseased ($n = 17$) from the analysis [37]. Seven of these 17 dolphins had orogenital neoplasms, a condition previously shown to be associated with an up-regulated immune response [38]. Sample size limitations precluded further subdividing the data. A p value < 0.05 was considered statistically significant. All analyses were conducted using SPSS Version 19.

Additional analyses were conducted for $\sum$PFCs of hematology and serum chemistry parameters by dividing exposure into tertiles. The adjusted mean values for the high and low tertiles were compared with previously published 90% confidence intervals (CI) for upper and lower threshold values to determine whether the data fell within reference values for southeastern coastal dolphins for each parameter [39].

RESULTS

PFC exposure

Concentrations of PFCs were determined in plasma samples from a total of 79 Charleston, South Carolina dolphins ranging in age from 3 to 33 years (mean, 14.1 years). There were totals of 33 males and 48 females; 37 were classified as juveniles and 44 as adults. The mean values (±SD) and ranges for $\sum$PFCs, $\sum$PFCA, $\sum$PFSA, PFOS, PFOA, PFDA are provided in Table 1. The mean $\sum$PFC concentration was $1{,}970 \pm 1{,}380$ ng/ml with a range of 574 to 8,670 ng/ml. Mean $\sum$PFCA and $\sum$PFSA concentrations were 204 ± 184 ng/ml (78% of total PFCs) and $1{,}530 \pm 1{,}080$ ng/ml (22% of total PFC), respectively. Of the PFC analytes measured, PFOS comprised 73% of the $\sum$PFC, with PFDA comprising 9% and PFOA comprising 3% of the $\sum$PFC, respectively.

Immunology

Age-adjusted linear regression analysis indicated associations between exposure to PFCs and several immunological parameters in Charleston, South Carolina dolphins as shown in Table 2 (note, only statistically significant parameters are included in the tables). The strongest associations were between increasing serum concentrations of ΣPFC and increasing numbers of CD21+ cells and the CD2/C21 ratio; increasing serum concentrations of PFOS and increasing numbers of CD21+ cells; and increasing concentrations of ΣPFSA and CD21+ cells. All of the significant associations observed for the age-adjusted data were positive. Serum concentrations of PFOS were also significantly associated with increases in CD2+, CD4+, CD19+, and MHCII+ cells, as well as B-cell proliferation and granulocyte and monocyte phagocytosis. In comparison, serum concentrations of PFOA were significantly associated only with increases in CD2+ cells and the CD2/CD21 ratio. The ΣPFSA were significantly associated with the same endpoints as PFOS, whereas ΣPFCA concentrations were positively associated with numbers of CD2+cells, B-cell proliferation, and serum IgG1concentrations.

Associations between immune parameters and serum PFOS concentrations following stratification by gender were more robust than in the age-adjusted main analysis as shown by uniformly higher r^2 values for all parameters (Table 3). Stratification by gender indicated that serum PFOS concentrations were significantly associated with B-cell proliferation in males, whereas serum PFOS concentrations were associated with CD2+, CD4+, CD19+, CD21+, MHCII+ cells, and granulocyte phagocytosis in females. No relationship was detected between any of the PFCs and natural killer cell activity, T-cell proliferation (Con A), lysozyme activity, or respiratory burst.

Hematology/serum chemistry

Statistically significant associations between exposure to PFCs and several hematology and serum chemistry variables were found in Charleston, South Carolina dolphins after adjusting for age (Table 4). Creatinine was positively associated with all the following PFCs: $\sum$PFC, $\sum$PFCA, $\sum$PFSA, PFOS (and PFOA, although this was not significant). Similarly, phosphorus was also positively associated with several PFCs: $\sum$PFCA, $\sum$PFSA, PFOS, and PFOA (and PFDA, although at $p < 0.06$). There was a positive association between amylase and $\sum$PFCA, PFOA, PFDA. Alanine aminotransferase was positively associated with $\sum$PFSA and PFOS concentrations (and $\sum$PFCA had $p < 0.06$), whereas ALT was negatively associated with PFDA. Positive associations were found between PFOS and gamma-glutamyltransferase (GGT), whereas negative associations were found between PFOS and cholesterol. The lactate dehydrogenase (LDH) levels were negatively associated with PFDA. The $\sum$PFCs were negatively associated with the absolute number of monocytes and negative associations were found between PFOS and eosinophils (a similar trend was observed with $\sum$PFCA at $p < 0.06$). Negative associations were observed between PFOA concentrations and creatinine phosphokinase and anion gap. Concentrations of $\sum$PFCA, PFOA, and PFDA were positively associated with serum amylase levels. Also, $\sum$PFC was negatively associated with chloride.

Table 2. Results of age adjusted linear regression analyses for plasma concentrations (ng/ml) of $\sum$perfluoroalkyl compounds (PFC), $\sum$perfluoroalkylsulfonates (PFSA), and $\sum$perfluoroalkylcarboxylates (PFCA), perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and perfluorodecanoic acid (PFDA) and immune parameters in dolphins from Charleston, South Carolina, USA[a]

Immune Test	$\sum$PFC r^2	$\sum$PFCA r^2	$\sum$PFSA r^2	PFOS r^2	PFOA r^2	PFDA r^2
CD2 T cells (absolute nos.) ($n = 61$)		+0.09[b]	+0.15*	+0.15*	+0.15*	
CD4 helper T cells (absolute nos.) ($n = 77$)			+0.10	+0.11*		+0.11*
CD 19 B cells-immature (absolute nos.) ($n = 77$)				+0.24		
CD 21 B cells-mature (absolute nos.) ($n = 77$)	+0.39*		+0.39*	+0.40*		
CD2/CD21 ratio ($n = 61$)	+0.42*				+0.29*	
MHCII+ (absolute nos.) ($n = 77$)			+0.25*	+0.26*		+0.22
B cell proliferation (LPS 120) ($n = 77$)		+0.30*	+0.23*	+0.22*		+0.22*
IgG1 (mg/ml)($n = 56$)	+0.23*	+0.29*				
Granulocytic phagocytosis ($n = 33$)	+0.17		+0.20*	+0.20*		+0.17*
Monocytic phagocytosis ($n = 32$)	+0.25					

[a] Only r^2 values with $p < 0.05$ are shown.
[b] (−), (+) = indicates slope direction.
* p value ≤ 0.01.

Table 3. Results of age adjusted linear regression analyses for plasma concentrations (ng/ml) of perfluorooctane sulfonate (PFOS) and hematology, serum chemistry, and immune parameters, by gender[a]

Variable	∑PFOS r^2 (slope) males	p value	∑PFOS r^2 (slope) females	p value
BUN (mg/dl) ($n=79$)	+0.27[b]	<0.01		
Creatinine (mg/dl) ($n=79$)			+0.15	0.03
Uric acid (mg/dl) ($n=79$)			−0.16	0.04
Alkaline phosphatase (U/l) ($n=79$)			+0.44	0.05
Cholesterol (mg/dl) ($n=79$)	+0.33	0.04		
Triglyceride (mg/dl) ($n=79$)	−0.19	0.01		
Eosinophils (10^3cells/ul) ($n=79$)			−0.23	<0.01
Mean corpuscular hemoglobin concentration (MCHC) (g/dl) ($n=79$)	+0.11	0.05		
CD2 T cells (absolute nos.) ($n=61$)			+0.22	0.03
CD4 helper T cells (absolute nos.) ($n=77$)			+0.29	0.01
CD19 B cells-immature (absolute nos.) ($n=77$)			+0.36	0.03
CD21 B cells-mature 9 (absolute nos) ($n=77$)			+0.45	0.01
MHCII+ (absolute nos.) ($n=77$)			+0.36	0.01
B cell proliferation ($n=77$)	+0.27	0.01		
Granulocytic phagocytosis ($n=33$)			+0.68	<0.01

[a] Only r^2 values with $p \leq 0.05$ are shown.
[b] (−), (+) = indicates slope direction.
BUN = blood urea nitrogen.

Serum protein electrophoresis results indicated significant positive associations between PFOA and total protein and total globulin concentrations. Other positive associations were shown between ∑PFCA and both total alpha-globulin and alpha-2 globulin and between ∑PFC and total beta globulin (Table 4). Negative associations were found between several PFCs (∑PFSA, PFOS, and ∑PFCA, PFDA) and alpha 1-globulin, and as well between PFOS and total alpha-globulin.

In age-adjusted analyses stratified by gender (Table 3), increases in exposure to PFOS were associated with significant increases in blood urea nitrogen (BUN), mean corpuscular hemoglobin concentration, and cholesterol, and a decrease in triglycerides in males. Among females, an increase in the concentration of PFOS was directly related to serum creatinine and alkaline phosphatase (ALP) ($p=0.06$) and a decrease in uric acid and the absolute number of eosinophils.

The data were re-analyzed after removing 17 dolphins classified as diseased ($n=64$) [37] to evaluate potential confounding by this variable. The removal included seven dolphins with orogenital papilloma, a neoplasm of viral origin shown to up-regulate the immune system [38]. The concentration of PFOS in diseased dolphins (1,399.4 ± 763.5) was not significantly different from those without disease (1,428.4 ± 1,120.1) by t test. The lack of an association between PFOS concentration and disease status was further confirmed by examining scatterplots of PFOS and key outcome variables, which showed the distribution of diseased dolphins to be random (data not shown). Similarly, the concentration of total PFCs was not statistically different ($p=0.28$) in diseased dolphins (2,204.7 + 1,307.4) compared to those without the disease (1,915.0 ± 1,399.9). Deleting the 17 diseased dolphins produced increases in the r^2 values for all immune parameters except granulocytic phagocytosis, for which the p value increased to 0.07. For total PFCs, the results were more variable. The granulocytic and monocytic cell phagocytosis were no longer significant. Conversely, the p value for B cell

Table 4. Results of age adjusted linear regression analyses for plasma concentrations (ng/mL) of ∑perfluorochemicals (PFC), ∑perfluoroalkylsulfonates (PFSA), and ∑perfluoroalkylcarboxylates (PFCA), perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and perfluorodecanoic acid (PFDA) and hematology and serum chemistry parameters in dolphins from Charleston, South Carolina, USA[a]

Parameter	∑PFC r^2	∑PFCA r^2	∑PFSA r^2	PFOS r^2	PFOA r^2	PFDA r^2
Chloride (mEq/l) ($n=79$)	−0.17* [b]					
Anion gap ($n=79$)					+0.10	
Creatinine (mg/dl) ($n=79$)	+0.24	+0.29*	+0.28*	+0.27*		
Phosphorus (mg/dl)($n=79$)		+0.10*	+0.11*	+0.10*	+0.27*	
Alanine aminotransferase (ALT) (U/l) ($n=79$)			+0.21*	+0.22*		−0.16
Lactate dehydrogenase (LDH) (U/l) ($n=79$)						−0.29
Creatine phosphokinase (CPK) (U/l) ($n=79$)					−0.08	
Amylase (U/l) ($n=79$)		+0.18			+0.16	+0.15
Gamma glutamyltransferase (GGT) (U/l) ($n=79$)				+0.09		
Cholesterol (mg/dl) ($n=79$)				−0.15*		
Monocytes (10^3cells/ul) ($n=79$)	−0.07					
Eosinophils (10^3cells/ul) ($n=79$)				−0.11*		
Total protein (g/dl) ($n=79$)					+0.29*	
Total globulin (g/dl) ($n=79$)					+0.36	
Total alpha globulin (g/dl) ($n=79$)		+0.13		+0.10		
Alpha-1 globulin (g/dl) ($n=79$)		+0.26	+0.15	+0.14		−0.12*
Alpha-2 globulin (g/dl) ($n=79$)		+0.16				
Total beta globulin (g/dl) ($n=79$)	+0.11*					

[a] Only r^2 values with $p < 0.05$ are shown.
[b] (−), (+) = indicates slope direction.
* p value ≤ 0.01.

proliferation decreased to <0.05. In summary, deleting the diseased dolphins from the data had little effect on the results for PFOS despite a reduced sample size and statistical power, whereas the results for ∑PFC were more variable.

Further analyses were conducted to determine whether the statistically significant associations detected were of biological or clinical significance (Table 5). The age adjusted means for hematology and serum chemistry parameters were divided into tertiles by ∑PFC exposure and the values compared to previously published reference values for wild dolphins [39]. The mean values for the upper exposure tertile of uric acid, alkaline phosphatase, albumin, total alpha globulin, and alpha-1-globulin fell within the 90% CI of the upper threshold value for each parameter. Conversely, the mean for the highest exposure tertile of total globulin fell within the 90% CI of the lower threshold value and the means for the highest exposure tertiles of alpha-2-globulin and total beta globulin fell below the 90% CI for the lower threshold value. In addition, the adjusted means for ALT, LDH, GGT, and cholesterol were less than 11% lower than the lower bound of the 90% CI for the upper threshold value.

DISCUSSION

The present study is the first report of health effects associated with exposure to PFC in wild cetaceans. The dolphins from the Charleston, South Carolina site are a highly exposed population with among the highest reported PFC concentrations in marine mammals [7], comparable to occupationally exposed humans [25]. The environmental sources of PFC compounds include consumer and industrial products, commercial use of PFC-containing products, and transformation of precursors. Surface waters, particularly oceans, have been implicated as a major sink for PFCs [40]. Although the sources of exposure for Charleston dolphins have not been characterized fully, a dolphin food web study found PFCs in wastewater treatment discharge into the Charleston Harbor, the surrounding estuarine waters, sediments, and fish [7]. The concentrations of PFDA were many times higher in the Charleston food web compared to Sarasota, Florida, USA (South Carolina sediment was 0.4 vs 0.0004 ng/g in Florida; fish ranged from 1.5 to 5.5 ng/g in South Carolina and were below detection in Florida). Perfluorooctane sulfonate has been the major analyte found in wastewater treatment discharges from urban environments, and PFOA, PFHxS, PFNA, PFDA, and PFOSA were also detected [41]. Studies have suggested that biodegradation of fluorotelomer-based materials within treatment plants may be a likely source of PFCAs into this environment. Furthermore, because individual PFCs have differing industrial sources, some point source discharges might exist that could include PFCAs and specific analytes such as PFDA. Nonpoint source pollution originating from urbanized sites is a key factor of PFC

Table 5. Age adjusted means for hematology, serum chemistry, and protein electrophoresis by total plasma perfluoroalkyl compounds (PFC) tertiles (ng/ml wet wt) in dolphins near Charleston, South Carolina, USA, with previously published reference ranges for bottlenose dolphins

Variable	Lowest exposure tertile (SE)	Highest exposure tertile (SE)	90% CI for lower threshold value[a]	90% CI for upper threshold value[a]
Hematology parameters				
Neutrophils (10^3 cells/ul)	4.29 (0.34)	3.86 (0.36)	2.3–2.8	6.9–10.7
Lymphocytes (10^3 cells/ul)	2.46 (0.25)	2.43 (0.27)	0.3–0.7	3.4–4.5
Monocytes (10^3 cells/ul)	0.27 (0.05)	0.22 (0.05)	NA	0.8–1
Eosinophils (10^3 cells/ul)	4.54 (0.45)	2.99 (0.47)	1.4–2.2	7.1–8.2
Basophils (10^3 cells/ul)	0.07 (0.03)	0.06 (0.03)	NA	0.3–0.6
Platelets (10^3/ul)	180.23 (14.05)	207.38 (14.83)	86–109	240–277
Serum chemistry parameters				
Glucose (mg/dl)	95.69 (2.54)	92.52 (2.68)	20–71	112–124
Sodium (mEq/l)	156.12 (0.61)	154.40 (0.64)	151–153	159–161
Potassium (mEq/l)	3.72 (0.08)	3.86 (0.09)	3.0–3.3	4.4–4.7
Chloride (mEq/l)	114.40 (0.70)	113.24 (0.74)	106–109	119–129
Total bilirubin (mg/dl)	0.09 (0.01)	0.09 (0.01)	NA	0.2–0.3
Direct bilirubin (mg/dl)	0.01 (0.01)	0.01 (0.01)	NA	0.1–0.1
Calcium (mg/dl)	9.42 (0.10)	9.43 (0.11)	8.5–8.7	10.0–10.6
Phosphorus (mg/dl)	4.67 (0.17)	5.10 (0.18)	2.5–3.6	6.3–7.0
Magnesium (mg/dl)	1.48 (0.03)	1.48 (0.03)	1.2–1.3	1.7–1.8
Uric acid (mg/dl)	0.24 (0.13)	1.21 (0.43)	0.1–0.1	0.9–2.3
Alkaline phosphatase (U/l)	270.61 (33.41)	328.90 (35.26)	51–59	274–454
Alanine aminotransferase (U/l)	27.87 (3.80)	55.75 (9.92)	14–19	59–82
Aspartate aminotransferase (U/l)	238.29 (16.73)	233.33 (17.66)	118–169	369–733
Lactate dehydrogenase (U/l)	377.69 (28.57)	503.00 (26.60)	325–351	512–596
Gamma glutamyltransferase (U/l)	23.40 (1.13)	26.79 (1.19)	11–16	30–38
Cholesterol (mg/dl)	137.29 (11.66)	204.52 (22.58)	88–114	224–237
Triglyceride (mg/dl)	93.85 (17.98)	62.25 (18.98)	35–41	122–162
Serum protein electrophoresis parameters				
Total protein (g/dl)	7.07 (0.09)	7.19 (0.10)	6.7–7.0	8.1–8.8
Albumin (g/dl)	4.44 (0.06)	4.38 (0.06)	2.9–3.1	4.0–4.4
Total globulin (g/dl)	2.63 (0.11)	2.81 (0.11)	2.6–3.3	4.6–5.3
A/G ratio	1.76 (0.11)	1.70 (0.12)	0.8–1.0	2.1–2.6
Total alpha globulin (g/dl)	0.88 (0.07)	1.80 (0.08)	1.2–1.2	1.7–1.8
Alpha-1 globulin (g/dl)	0.23 (0.05)	0.73 (0.06)	0.1–0.2	0.5–1.1
Alpha-2 globulin (g/dl)	0.49 (0.05)	0.41 (0.05)	0.6–0.8	1.3–1.4
Total beta globulin (g/dl)	0.11 (0.03)	0.17 (0.03)	0.3–0.4	0.6–0.7
Gamma globulin (g/dl)	1.63 (0.13)	1.54 (0.14)	1.0–1.4	2.5–3.6

[a] Confidence intervals for low and high reference values from Schwacke [39].
SE = standard error; CI = confidence interval; NA = not available.

contamination in aquatic ecosystems [42]. In addition to non-point sources, the high PFC levels found in these dolphins suggest a high likelihood of point source(s) in this region, which remain to be elucidated. Although there are no recognized point sources in Charleston, studies are in progress to further characterize PFCs in this environment. Because Charleston dolphins exhibit site fidelity they forage, reproduce, and nurture young in one of the more urbanized ecosystems, serving as good indicators of environmental contamination and sentinels for potential health effects.

In the present study, we aimed to determine whether serum PFC concentrations in dolphins using total PFC, the PFCA and PFSA groups, as well as individual analytes (i.e., PFOS, PFOA, and PFDA) were associated with markers of health status in the highly exposed dolphin population inhabiting the estuarine waters of Charleston, South Carolina. Despite the ubiquity of PFCA and PFSA contamination, the sources of exposure and their effects are not fully known, although studies have shown varying biochemical and transcriptional responses related to PFC isomer and chain length [43]. We found multiple statistically significant associations between plasma PFC concentrations and hematologic, biochemical, and immunologic parameters. In the present study, the majority of the associations between PFC and immune and hematology parameters were observed for the $\sum$PFSA group, which comprised 75% of the total PFCs and PFOS as the major analyte.

To date, assessments of immune function in relation to PFCs have been conducted in rodent and chicken models [13,14]. Epidemiological studies in humans have indicated that PFCs are associated with decreased antibody titers to common childhood vaccines, but that PFOS and PFOA are not associated with increased risk of hospitalization from infectious diseases [16,44]. Studies in humans living near PFOA production facilities have reported negative associations between serum PFOA concentrations and IgA, IgE, and C-reactive protein, positive relationships with anti-nuclear antibodies, and no association with IgG (C8 science panel, http://www.c8sciencepanel.org/pdfs/Status_Report_C8_and_Immune_markers_March2009.pdf).

The present study observed few associations between PFOA and immunological biomarkers, but noted multiple associations with PFOS that were mirrored in the $\sum$PFSA associations, suggesting it may be the primary contributor to the observed $\sum$PFSA associations. Associations between $\sum$PFCA and numbers of peripheral blood CD2+ cells and B-cell proliferation were observed and were also shown for at least one of the individual PFCs. Fewer associations between ΣPFC and immune parameters were found than for PFOS and ΣPFSA and did not seem related, for the most part, to other significant associations for the classes or single compounds. For example, the numbers of CD2+ cells were weakly associated with increasing PFOA, PFOS, PFCA, and PFSA, but ΣPFC was not significantly associated with the numbers of CD2+ cells. Combining PFCs when assessing effects might not be the most appropriate approach due, in part, to varied results on specific endpoints with different PFCs and possible differences in mechanisms [45]. Thus, for immune function, it seems that PFOA may act in PPAR-α–dependent manner, whereas PFOS does not [13].

In the present study, PFOS was noted to be significantly and positively associated with the numbers of CD2+, CD4+, CD19+, CD21+, and MHCII+ cells and with B-cell proliferation and granulocyte phagocytosis. Moreover, when stratified by gender, female dolphins exhibited significant associations with CD2+, CD4+, CD19+, CD21+, and MHCII+ cells. Differences in response to PFOS on the immune system between sexes have also been described in laboratory animals [36,46]. The only other study to date to have assessed a relationship between MHCII+ cells and PFOS exposure reported no effect on the numbers of cells expressing MHCII in female mice [47]. It is important to note, however, that MHC class II expression levels are higher in cetaceans than humans and rodents and are not restricted to classical antigen presenting cells (i.e., macrophage/monocyte, B-cell, and dendritic cell), such that almost 100% of peripheral T-cells have been reported to express high levels of MHCII [48].

Some mouse studies have shown slight increases in splenic CD4+ cells that were not statistically significant in several studies at mean PFOS serum concentrations of 17 ppb to 674 ppb in male mice [36,49]. At higher mean PFOS serum concentrations, ranging from 21 to 338 ppm, numbers of splenic CD4+ cells were decreased [49,50]. Only one study has assessed peripheral blood CD4+ cell numbers following PFOS exposure. In rats, no effect on absolute numbers of peripheral blood CD4+ cells were reported at mean serum PFOS concentrations up to 43 ppm [46]. Similarly, numbers of splenic B220+ cells (a mouse B-cell marker) showed slight increases in several mouse studies at PFOS serum concentrations ranging from 12.5 to 110 ppm [47,49,50], whereas decreases were noted in B220+ cells at mean PFOS serum concentrations ranging from 21 to 338 ppm [49,50]. Lefebvre et al. [46] reported no alteration in numbers of peripheral blood CD45RA+ cells (B-cell marker) in rats at serum PFOS concentrations up to 43 ppm. Taken together, mouse data suggests trends of increasing numbers of CD4+ cells and B-cells somewhat similar to the observations noted with the dolphins in the present study.

B-cell proliferation in the dolphins, which was significantly and positively associated (albeit weakly) with PFOS serum concentrations, stratification by sex indicates this was due primarily to a relationship in male dolphins but not female dolphins. Dong et al. [49] observed a slight increase in B-cell proliferation in male mice exposed to PFOS for 60 d at the lowest dose tested (mean PFOS serum concentration = 674 ppb). However, B-cell proliferation in male and female mice and rats following a 28-d exposure was not altered at mean serum concentrations of 12 to 43 ppm, respectively [36,46]. Zheng et al. [50] reported decreased B-cell proliferation in male mice following a 7-d exposure with mean serum PFOS concentrations of 110 ppm to 388 ppm. These data suggest that longer exposure to lower daily concentrations could be related to increased B-cell proliferation. However, data from studies with peripheral blood leukocytes from bottlenose dolphins, exposed in vitro to a range of PFOS concentrations representative of those found in Charleston, South Carolina dolphins fit a standard dose–response model and exhibited a statistically significant, increasing trend in proliferation in relation to PFOS dose (J. Wirth, 2012, Master's Thesis, College of Charleston, Charleston, SC, USA).

Perfluorooctanoic acid was significantly associated with only the numbers of CD2+ cells and the CD2/CD21 ratio, whereas ΣPFCA concentrations were associated with the numbers of CD2+cells, B-cell proliferation, and IgG titers. For the specific parameters assessed, few studies exist in the literature for comparison with PFOA. The CD2 is a general marker for T-cells, and no studies in the literature have assessed this in relation to PFOA. However, sub-populations of T-cells have been assessed. Yang et al. [51] reported decreases in CD4+ and CD8+ cell types in the spleen and thymus following PFOA

exposure. Son et al. [52] reported increases in the numbers of splenic CD4+ cells and decreases in the numbers of splenic CD8+ populations. Perfluorooctanoic acid exposure in mice has been shown to result in increased serum levels of antigen-specific-IgG [53]. As previously noted, the observed association between ΣPFCA and serum total IgG titers was not attributed to any of the single compounds included in the present summation, possibly suggesting a combined action through a similar mechanism in relation to IgG production. This warrants further investigation.

Phagocytosis, a component of the innate immune response, plays an integral role in the host's defense against invading microorganisms and is a component of the tissue response in inflammation and necrosis. In Charleston, South Carolina, dolphins with high PFC exposures, positive associations were found with both monocytic and granulocytic phagocytosis, particularly for PFOS exposure in males with an r^2 value of 0.68. Phagocytic activity was found to be sensitive to toxicants in rodents [54] and has been suggested as an assessment tool to monitor exposure to chemicals in wildlife species [55].

Several studies have assessed immune modulation in laboratory animals following PFC exposure. Evidence is mounting for the immunotoxic effects of these compounds, although the results are not entirely consistent. For a given chemical, the response of the immunological markers can be contradictory in some cases as effects can range from immunosuppression to immunostimulation, or no effect at all, depending on factors including species, mode, and duration of exposure, dose [56]. It should also be noted that wild populations are not exposed to single PFCs, which is in contrast to controlled animal studies. In addition, limited research exists on the effects of PFC exposure on the immune system in animals. Furthermore, the dolphin immune system is largely uncharacterized compared to laboratory animals; thus, interpreting the results has inherent limitations. Differences observed between studies in laboratory animals and wildlife may be a result of various factors, including species variability, exposure dose and duration, mixtures and compositions of PFCs, differences in exposure patterns, and potential confounders such as concomitant exposures to other toxicants. Further work is needed to confirm these relationships, and longitudinal analyses can help determine whether high PFC exposures predict future onset of disease.

Both animal studies and evaluations of workers have shown effects on the liver and serum lipid profiles [1]. Liver injury in humans and animals, including dolphins, is typically assessed using ALP, ALT, and AST biomarkers [57]. In the present study, age-adjusted regression analyses showed that several markers of hepatic function in dolphins were associated with plasma PFC concentrations. Positive associations between concentrations of PFCs and serum GGT and ALT concentrations were observed. Serum GGT levels were associated with increasing PFOS concentrations, whereas ALT levels were associated with increasing concentration of $\sum$PFSA and PFOS (also, $\sum$PFCA had $p = 0.06$). With the combined gender analysis, no relationship was observed between PFOS concentration and ALP; however, in female dolphins, increasing PFOS was significantly associated with increasing ALP levels. Both occupational exposures in humans and animal dosing studies have shown PFC exposures are associated with higher levels of the hepatic enzyme markers of liver damage.

Elevated exposures to PFOS and/or PFOA in both occupationally exposed humans [58] and the general population [22,23,59] have generally been associated with increases in liver enzymes. Perfluorooctanoic acid and PFOS were associated with ALP and ALT in workers [23], although another study of the general population found associations only with PFOA [58]. The association between PFOA and PFOS and GGT in human studies has been inconsistent [21,22,59]. Elevated GGT likely indicates intrahepatic cholestasis and occurs at an early stage and is more persistent than ALP in cholestatic disorders [60]. Acute and chronic toxicity studies in rodents have described morphological and biochemical alterations in the liver following exposure to PFCs. The liver toxicity of several PFCs is also evidenced by lipid accumulation and vacuolization in monkeys and rodents [10,61]. Clinical chemistry changes have been reported following exposure to PFOS and PFOA in rodents with increases in ALT, AST, and AP [11,62]. One study in rodents reported increases in liver weight, ALT, and BUN, with males being more sensitive to the hepatocellular effects of PFOS exposure [4]. The observed positive associations in dolphins between serum enzymes GGT, ALT, and ALP with PFC concentrations are consistent, generally, with those reported in animal and occupational workers in terms of direction. In dolphins, increases in ALT may reflect heptocellular necrosis, neoplasia, parasitism, or hepatic/muscular trauma, whereas ALP levels vary with age, nutritional status, and infection or inflammation [63].

Cholesterol levels in the dolphins were weakly associated with PFOS levels in age-adjusted regressions. In gender-stratified analyses, however, cholesterol and triglyceride were found to be negatively associated with PFOS in males only. The increased cholesterol in combined male and female dolphins associated with PFC contradicts what has been reported from animal studies. Instead, it is more closely related to findings from occupationally exposed workers [64–66] and a recent study in humans with environmentally relevant exposures to PFOS, PFOA, and PFNA [67]. However, the negative association between cholesterol and PFOS in male dolphins shown in Table 3 was stronger and is consistent with numerous animal studies showing a hypolipodemic effect of PFCs. Interestingly, studies in rodents generally found lower cholesterol levels with PFOS and PFOA concentrations, although the doses typically were higher than human exposure levels [68–70]. Also, decreased cholesterol was reported in cynomolgus monkeys following PFOS exposure [10] but not with PFOA exposure [71]. In rats, cholesterol decreased in dams following PFC exposures but increased in fetuses [72]. Seacat et al. [11] found a significant decrease in cholesterol in male rats after four weeks, but this was not evident in females.

Serum creatinine concentrations were associated positively with all of the PFC groups, except PFDA in Charleston, South Carolina dolphins. When separated by gender, creatinine was associated with PFOS exposure only in females. Creatinine, a by-product of muscle metabolism, is excreted by the kidneys and levels increase when filtering by the kidney is deficient. Results from several studies lend support for the kidney as a target organ for PFCs [73]. The primary route of excretion is through the kidneys in monkeys [74], and urine has also been demonstrated to be a route of PFC excretion in dolphins (M. Houde, Environment Canada, personal communication). Furthermore, greater concentrations of PFOS and PFOA in the kidneys have been reported [75], in addition to renal hypertrophy and histopathologic changes and renal microvascular disease in rats exposed to PFCs [76]. Chronic kidney disease (CKD) is a major public health problem, and the results from experimental animal studies have suggested that an association between PFCs and CKD is plausible [9]. A recent study examined the relation of serum PFCs and CKD in more than

4,500 adults from the combined National Health and Nutritional Examination Survey and found associations between higher serum levels of PFCs, including PFOA and PFOS, and CKD [77]. Although PFOS is considered more biologically active [6], this study found both PFOS and PFOA equally associated with CKD markers. In marine mammals, serum creatinine is considered a crude index of glomerular filtration and is not markedly influenced by diet [63]. Gender-specific analysis indicated a positive association between PFOS concentrations and increased BUN only in male dolphins.

Serum phosphorus was also positively associated with several of the PFC groups ($\sum$PFCA, $\sum$PFSA, PFOS, PFOA, and PFDA). Progressive renal insufficiency leads to hyperphosphatemia [78], and increased creatinine with elevated phosphorus indicates long-standing kidney disease. The findings of both creatinine and phosphorus associations with PFCs in the dolphins and the consistency in the gender-stratified subgroup analysis suggests that these findings are less likely due to chance.

The absolute number of eosinophils significantly decreased with increasing concentrations of PFOS and a similar trend was observed with $\sum$PFCA ($p < 0.06$). A decrease in the number of eosinophils may interfere with the functions of these cells in physiological and pathological processes [79]. The decrease in eosinophils was weakly associated with PFC exposure for both sexes combined and was stronger and significant only in females. It is possible that exposure to PFCs and/or other chemicals may interfere with eosinophil-mediated processes, including glucocorticoid-induced regulation of an immune response. Leukogram changes in mice following PFOA exposures (30mg/kg/d) were also characterized by significant decreases in eosinophils [70], similar to our findings in dolphins.

Separating protein serum fractions by electrophoresis is important for diagnosing different diseases in humans and marine mammals [63,80]. Significant associations were found between PFC levels and several serum proteins in dolphins. Significant positive associations were observed between PFOA and total protein and total globulin concentrations. Total beta globulin also increased with increasing $\sum$PFC. Positive associations also were shown between $\sum$PFCA concentrations and both total alpha-globulin and $alpha_2$-globulin. Negative associations were found between several PFCs and $alpha_1$-globulin and PFOS and total alpha-globulin. Alpha-globulins are acute-phase proteins that include haptoglobulin, lipoproteins, antitrypsin, ceruloplasmin, and $alpha_2$-macroglobulin that may contribute to this increase [81] and are important early markers of acute inflammatory disease.

The present study has several limitations that dictate caution in interpreting the results. The cross-sectional design did not afford an opportunity for longitudinal follow-up over time. Thus, the duration of changes in the affected parameters could not be assessed, nor could the temporality of the associations be determined. Although many statistically significant associations were found, the degree of variation may not have been adequate to be of clinical significance, and some associations may have been due to chance. A sensitivity analysis was conducted of $\sum$PFCs, and PFOS were not significantly higher in diseased dolphins using a previously published system of classification [37]. Nonetheless, many of the associations reported here were supported by the finding that their values for the highest tertile of exposure to $\sum$PFCs fell within or approached 10% of the confidence limit for the upper threshold of values from four southeastern coastal reference populations of dolphins [39]. Similarly, in occupationally exposed human populations, changes in biochemical parameters have been demonstrated in the absence of overt disease [20].

Confounding effects due to other contaminants known to exist in the Charleston, South Carolina dolphins may have been responsible for some of the associations detected. Previous studies have shown high concentrations of other organic chemicals, including polybrominated diphenyl ethers, polychlorinated biphenyls, and pesticides in Charleston dolphins [82,83], which could cause confounding if the chemical is related to both PFC exposure and the outcome. Despite these limitations, the present study has several strengths, including a relatively large sample size for a wildlife study allowing an exploration of effect modification by sex. The consistency of the findings with the literature on PFC suggests that the associations reported in this first study of wild dolphins exposed to relatively high PFC concentrations are likely to be real. The levels of PFC exposure in the dolphin population studied are markedly above those reported in other marine mammals [6] and similar to those in occupationally-exposed humans [25].

CONCLUSION

The present study evaluated a free-ranging population of dolphins with chronic exposures to high levels of PFCs and found multiple associations between serum PFC concentrations and stimulation of both innate and adaptive immunity (both humoral and cell-mediated), along with modulation of various clinicopathologic parameters including liver enzymes. These findings indicate that the dolphins' chronic exposure to these contaminants appear to be producing immune perturbations and tissue toxicity. Further research is needed to clarify long-term effects. To our knowledge, this is the first study to demonstrate associations between serum PFC concentrations and immune, hematological, and biochemical markers in marine mammals. The majority of the observed associations are in accordance with reported findings in other laboratory animals. Although none of these relationships establish causality, they do provide the first line of evidence that PFCs may be involved with altering immune, hematopoietic, renal, and hepatic function in wild dolphins with high PFC contaminant burdens.

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